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Persistently Infected Horses Are Reservoirs for Intrastadial Tick-Borne Transmission of the Apicomplexan Parasite *Babesia equi*[▽]

Massaro W. Ueti,[1]* Guy H. Palmer,[1] Glen A. Scoles,[2]
Lowell S. Kappmeyer,[2] and Donald P. Knowles[1,2]

Program in Vector-Borne Diseases, Department of Veterinary Microbiology and Pathology, Washington State University, Pullman, Washington 99164-7040,[1] and Animal Disease Research Unit, USDA Agricultural Research Service, Pullman, Washington 99164-6630[2]

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Tick-borne pathogens may be transmitted intrastadially and transstadially within a single vector generation as well as vertically between generations. Understanding the mode and relative efficiency of this transmission is required for infection control. In this study, we established that adult male *Rhipicephalus microplus* ticks efficiently acquire the protozoal pathogen *Babesia equi* during acute and persistent infections and transmit it intrastadially to naïve horses. Although the level of parasitemia during acquisition feeding affected the efficiency of the initial tick infection, infected ticks developed levels of $\geq 10^4$ organisms/pair of salivary glands independent of the level of parasitemia during acquisition feeding and successfully transmitted them, indicating that replication within the tick compensated for any initial differences in infectious dose and exceeded the threshold for transmission. During the development of *B. equi* parasites in the salivary gland granular acini, the parasites expressed levels of paralogous surface proteins significantly different from those expressed by intraerythrocytic parasites from the mammalian host. In contrast to the successful intrastadial transmission, adult female *R. microplus* ticks that fed on horses with high parasitemia passed the parasite vertically into the eggs with low efficiency, and the subsequent generation (larvae, nymphs, and adults) failed to transmit *B. equi* parasites to naïve horses. The data demonstrated that intrastadial but not transovarial transmission is an efficient mode for *B. equi* transmission and that persistently infected horses are an important reservoir for transmission. Consequently, *R. microplus* male ticks and persistently infected horses should be targeted for disease control.

The maintenance of tick-borne infections in natural reservoir hosts is dependent upon the efficiency of acquisition and transmission events at the tick-host and tick-pathogen interfaces (7, 20). Effective control of tick-borne infectious disease requires knowledge of the ability of specific vector stages to acquire, amplify, and transmit the pathogen. There are three nonmutually exclusive modes of tick-borne transmission (4). The first mode is transstadial transmission that occurs when a tick stage (e.g., larval or nymphal) acquires the pathogen from a mammalian reservoir host and a subsequent life cycle stage within the same tick generation transmits the pathogen to an uninfected host (11, 18, 20). In the second mode, ticks transmit the pathogen intrastadially; the pathogen is acquired by the tick, and following movement between individual animal hosts, the same tick stage transmits the pathogen to a naïve animal (18). In the third mode, transovarial passage is followed by pathogen transmission by one or more stages in the subsequent generation (3, 7). All three modes occur in the transmission of the apicomplexan parasites of the genus *Babesia* (4); however, the mode used by a given *Babesia* sp. cannot be inferred but, rather, requires testing and quantification of the efficiency in vivo.

Babesia equi infects equids and is transmitted throughout subtropical and tropical regions of the Americas by *Rhipicephalus* (*Boophilus*) *microplus* (6, 11). Transstadial transmission of *B. equi* parasites has been confirmed; *R. microplus* nymphs acquire *B. equi* infections during acquisition feeding on either acutely or chronically infected horses and, following molting and movement to a new host, can successfully transmit *B. equi* parasites to naïve horses (20). In contrast, whether *B. equi* parasites can be transmitted intrastadially or transovarially by *R. microplus* is unknown. In the first part of this study, we tested the hypothesis that adult male *R. microplus* ticks acquire and transmit *B. equi* parasites. Furthermore, we determined whether the parasitemia during acquisition feeding of the ticks affects the subsequent efficiency of tick infection. In the second part, we proceeded to test whether *B. equi* can be passed transovarially and transmitted by larvae, nymphs, or adults of the subsequent generation. Although vertical passage from adult females to eggs has been previously reported (2), actual transmission by tick stages of the next generation has not been tested. Herein, we report the testing of both hypotheses and discuss the epidemiologic significance of the results.

MATERIALS AND METHODS

Intrastadial transmission of *B. equi* parasites. To test the ability of adult male *R. microplus* ticks to acquire and transmit *B. equi* parasites intrastadially, horses were infected with the Florida strain of *B. equi* by either feeding infected *R. microplus* to them (horses H072 and H077) or by intravenous inoculation (horses H078 and H099). Approximately 20,000 larvae from 1 gram of eggs were allowed to feed on Holstein calves for 14 days to rear adult male ticks (5, 17). Engorged

* Corresponding author. Mailing address: Department of Veterinary Microbiology and Pathology, Washington State University, Pullman, WA 99164-7040. Phone: (509) 335-6325. Fax: (509) 335-8328. E-mail: massaro@vetmed.wsu.edu.

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nymphs were forcibly removed by using microsurgery forceps and incubated for 3 days at 26°C, at 94% relative humidity, and with a 12-h photoperiod to allow molting to the adult stage (17, 18). For acquisition feeding, approximately 250 freshly molted adult male *R. microplus* ticks were allowed to feed under a cloth patch for 9 days on horses during either acute infection (horses H078 and H099) or after long-term persistent infection (at 15 months postinfection; horses H072 and H077). Following acquisition feeding, adult male ticks were forcibly removed and incubated for 3 days at 15°C, at 94% relative humidity, and with a 12-h photoperiod to prevent any possible mechanical transmission of *B. equi* parasites by tick mouthpart contamination (17). Subsequent to incubation, for transmission feeding, approximately 100 adult male ticks were applied under a cloth patch and allowed to feed on four naïve horses (H085, H090, H098, and H112) to determine their ability to transmit *B. equi* parasites (18, 20). Following 8 days of transmission feeding, all male ticks were forcibly removed, and their individual salivary glands were dissected (20).

Genomic DNA was extracted from individual tick salivary glands (Gentra Systems, Inc., Minneapolis, MN) and tested by duplex nested PCR, targeting the *B. equi ema-1* and *R. microplus* α-*tubulin* genes (19, 20). Tick infection rates were calculated as previously described (7, 20) by dividing the total number of PCR-positive ticks by the total number of ticks tested. The levels of *B. equi* organisms in the peripheral blood of horses exposed to acquisition feeding and in the salivary glands of *R. microplus* ticks were quantified by real-time PCR, targeting *B. equi ema-1* as previously described (19, 20). The infection status of horses exposed to transmission feeding by adult male ticks was determined by Giemsa-stained blood smear and confirmed by nested PCR, targeting *B. equi ema-1* (20).

Detection and characterization of EMA-1 and EMA-2 paralog expression within the salivary glands of infected adult male ticks. A cohort of adult male ticks acquisition fed on acutely infected horses and transmission fed on naïve horses was dissected, and their salivary glands were examined by immunoblotting and by immunohistochemistry assays. Relative levels of equi merozoite antigen 1 (EMA-1) and EMA-2 expression were determined by immunoblotting *B. equi* antigens from adult male tick salivary glands and parasitized equine erythrocytes collected during the acute phase of infection (mean of $10^{6.1}$ *B. equi* parasites/ml of blood). Approximately 10^5 *B. equi* parasites, as quantified by real-time PCR, from infected tick salivary glands and equine erythrocytes were loaded per lane, and proteins were separated by electrophoresis and transferred to a nitrocellulose membrane. *B. equi* monoclonal antibody (MAb) 36/133.97 was used in immunoblots to determine the relative expression levels of the paralogs EMA-1 and EMA-2 (12, 19). An isotype-matched control antibody (18.185) which binds *Cryptosporidium parvum* was utilized as a negative control (19). Densitometry analysis of replicate samples ($n = 4$) was done on both *B. equi* intraerythrocytic and salivary gland stages. To localize *B. equi* colonies within the acini of salivary glands, tick salivary glands concentrated in 1% agarose were fixed in 10% formaldehyde and embedded in paraffin. Serial 4-μm sections were stained with 1 μg of MAb 36/253.21, which binds specifically to *B. equi* EMA-2 and to MAb 18.185 as a negative control as previously described (19). Following the incubation of the primary antibodies, the secondary antibody, horseradish peroxidase-labeled anti-mouse antibody (Dako Corp., Carpinteria, CA), was incubated with the salivary gland sections, and binding was detected using the substrate 3-amino-9-ethylcarbazole containing hydrogen peroxide (19). Sections were counterstained with Mayer's hematoxylin.

Transovarial transmission of *B. equi* parasites. To test the transovarial transmission of adult female *R. microplus* ticks and their offspring, approximately 20,000 *R. microplus* larvae from 1 gram of eggs were applied under a cloth patch to splenectomized horses (H061 and H144). When approximately 1% of the nymphs molted to adulthood, 8×10^7 *B. equi*-infected erythrocytes were inoculated intravenously into horses H061 and H144 to synchronize ascending *B. equi* parasitemia with repletion of female *R. microplus* ticks. Engorged female ticks that detached during ascending parasitemia were collected and incubated at 26°C, at 94% relative humidity, and with a 12-h photoperiod to allow for egg production (7, 8). To detect the most heavily infected female ticks with the highest likelihood of successful vertical transmission, an aliquot of hemolymph was collected from individual live ticks in cell lysis solution (Gentra Systems, Inc., Minneapolis, MN) with proteinase K (2 mg/ml) and tested for the presence of *B. equi* organisms by nested PCR. Genomic DNA samples were extracted as previously described (7), and nested PCR targeting *ema-1* was performed to determine the *B. equi* infection status (20). Following 10 days of egg production, portions of egg masses (approximately 100 eggs) were collected from individual adult female ticks, DNA was extracted, and infection was determined by *ema-1* nested PCR. Based on *B. equi* infection status as determined by nested PCR, egg masses were scored either positive or negative and pooled by this status. Pooled egg masses were incubated at 26°C, at 94% relative humidity, and with a 12-h photoperiod for hatching into larvae.

TABLE 1. *B. equi* infection rate and level within salivary glands of adult male *R. microplus* ticks acquisition fed on acutely and chronically infected horses

Horse for acquisition feeding[a]	No. of *B. equi* parasites/ml of blood during acquisition feeding (mean ± SD)	Horse for transmission feeding	% Tick infection rate (no. of ticks infected/ no. tested)	No. of *B. equi* parasites/ tick salivary gland pair (mean ± SD)
H078	$10^{6.4 \pm 0.47}$	H098	87 (21/24)	$10^{5.5 \pm 0.54}$
H099	$10^{6.0 \pm 0.64}$	H085	71 (21/29)	$10^{4.9 \pm 0.91}$
H072	$10^{5.2 \pm 0.53}$	H112	7 (2/28)	$10^{4.2 \pm 1.63}$
H077	$10^{5.3 \pm 0.43}$	H090	50 (8/16)	$10^{4.7 \pm 0.90}$

[a] Horses H078 and H099 were acutely infected, while H072 and H077 were chronically infected.

To determine whether a subsequent generation from adult female *R. microplus* ticks allowed to feed on horses with ascending parasitemia had the ability to transmit *B. equi* organisms, four splenectomized horses were utilized for transmission feeding. Horses H080 and H143 received approximately 10,000 and 14,000 larvae, respectively, hatched from PCR-positive eggs. Horses H142 and H060 received approximately 10,000 and 20,000 larvae, respectively, from PCR-negative eggs. The ticks were allowed to feed through all three life stages of *R. microplus* (larvae, nymphs, and adults) on the naïve, splenectomized horses to determine their ability to transmit *B. equi* parasites. The infection status of the splenectomized horses was determined by daily microscopic examination of Giemsa-stained blood smears and nested PCR targeting *B. equi ema-1* (20).

RESULTS

Intrastadial transmission of *B. equi* parasites. Adult male *R. microplus* ticks successfully acquired and intrastadially transmitted *B. equi* parasites to naïve horses. The level of *B. equi* parasites in the peripheral blood of acutely and chronically infected horses during 9 days of acquisition feeding showed that adult male ticks were exposed to approximately 10 times more parasites during the acute phase than during the chronic phase of infection (Table 1). The mean levels of *B. equi* parasites in acutely infected horses (H078 and H099) and in chronically infected horses (H072 and H077) were $10^{6.2}$ and $10^{5.3}$ per ml of blood, respectively. The percentages of adult male ticks which acquired infection were 87% (H078) and 71% (H099) of ticks allowed to feed on acutely infected horses and 7% (H072) and 50% (H077) of male ticks allowed to feed on chronically infected horses (Table 1).

To confirm tick salivary gland infection, the salivary glands from a cohort of adult male ticks were tested by immunoblot assay alongside infected blood samples collected during acquisition and transmission feeding as controls. *B. equi* parasites from both the blood and the tick salivary glands were detected by expression of EMA-1 and EMA-2 (Fig. 1). Densitometric analysis showed a difference in EMA-1 and EMA-2 expression levels between erythrocytic and salivary gland *B. equi* stages (Fig. 1). A comparison of equivalent numbers of *B. equi* parasites from equine erythrocytes and tick salivary glands revealed that *B. equi* erythrocytic stages expressed EMA-1/EMA-2 ratios of 2.7 (±0.17):1, which was significantly greater than a 1:1 ratio ($P = 0.029$, Mann-Whitney rank sum test). In contrast, *B. equi* salivary gland stages expressed EMA-1/EMA-2 ratios of 1:2.1 (± 0.71), which was significantly less than a 1:1 ratio ($P = 0.018$). The significantly lower ratio in *B. equi* salivary gland stages reflected lower levels of EMA-1 than

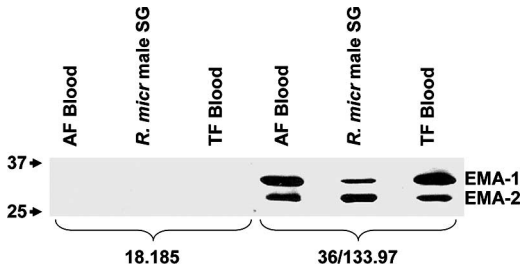

FIG. 1. Differential expression of EMA-1 and EMA-2 during *Babesia equi* infection within the mammalian host and tick vector. AF Blood, blood from infected horse at time of male tick acquisition feeding; *R. micr* male SG, infected adult male *Rhipicephalus microplus* ticks; TF Blood, blood from infected horse following transmission by adult male tick feeding; 18.185, isotype-matched control MAb; 36/133.97, MAb specific to *B. equi* EMA-1 and EMA-2. The positions of the 25- and 37-kDa molecular size markers are shown on the left.

those in the erythrocytic stages, while the EMA-2 levels appeared to be relatively constant (Fig. 1). To localize *B. equi* parasites within the salivary glands, sections were probed with MAbs reactive to EMA-2 by immunohistochemistry assay. Colonies were detected exclusively in the granular acini of salivary glands (Fig. 2). No reactivity was observed in sections of salivary glands from the same ticks incubated with an isotype-matched control antibody (Fig. 2).

The ability of *B. equi*-infected male ticks to transmit the parasites was evaluated by tick feedings on naïve horses. Adult male ticks acquisition fed on *B. equi*-infected horses H078, H099, H072, and H077 were transmission fed on naïve horses H098, H085, H112, and H090, respectively. Following 8 days of transmission feeding, the level of babesial parasites within the tick salivary glands ranged from $10^{2.6}$ to $10^{6.5}$ (mean of $10^{5.1 \pm 0.86}$ *B. equi* parasites per pair of salivary glands). All four naïve horses (H085, H090, H098, and H112) were shown to be infected with *B. equi* parasites by 10 days after tick exposure as determined by microscopic examination of Giemsa-stained blood smears and confirmed by nested PCR (data not shown). Notably, two infected adult male *R. microplus* ticks with a mean of $10^{4.2}$ parasites per salivary gland pair were sufficient to transmit the parasites to a naïve horse (Table 1).

Transovarial transmission of *B. equi* parasites. Adult female *R. microplus* ticks were allowed to feed to repletion on acutely infected horses, with *B. equi* levels in the peripheral blood starting at $10^{3.7}$ parasites/ml and progressing to $10^{9.2}$ parasites/ml of blood (H061) and starting at $10^{3.8}$ parasites/ml and progressing to $10^{9.4}$ parasites/ml of blood (H144). The percentages of hemolymph PCR-positive adult female ticks allowed to feed on horses H061 and H144 were 0.8% (4/493) and 1.5% (7/462), respectively. Subsequently, the percentages of *B. equi* PCR-positive egg masses from adult female ticks allowed to feed on horses H061 and H144 were 0.2% (1/493) and 2.1% (10/462), respectively.

The ability of the next generation of *R. microplus* ticks to transmit *B. equi* parasites was tested by allowing all stages of the offspring (larvae, nymphs, and adults) of *R. microplus* to transmission feed on naïve, splenectomized horses. Larvae from PCR-positive eggs were allowed to feed on horses H080 and H143, while larvae hatched from PCR-negative eggs were allowed to feed on horses H142 and H060. All three stages of *R. microplus* were allowed to feed on the same horse to determine their abilities to transmit *B. equi* parasites: larvae fed for 7 days and molted to nymphs, nymphs reattached and fed for 8 days and molted to adults, and adults reattached and fed for 7 to 11 days. None of the four splenectomized horses (H060, H080, H142, and H143) showed evidence of *B. equi* infection for 120 days after tick transmission feeding, as determined by microscopic examination of Giemsa-stained smears and nested PCR (data not shown).

DISCUSSION

The current studies establish that adult male *R. microplus* ticks efficiently acquire *B. equi* parasites during feeding on both acutely and chronically infected horses and transmit the organisms to naïve horses. The feeding and host-seeking behavior of adult male *R. microplus* ticks as well as the development of the parasite within the ticks support the epidemiologic significance of intrastadial transmission (13, 14). Although *R. microplus* is a one-host tick, meaning that larvae, nymphs, and adults can feed to repletion and molt on an individual host, adult male ticks feed intermittently with movement among individual mammalian hosts in search of female ticks (13). Consequently,

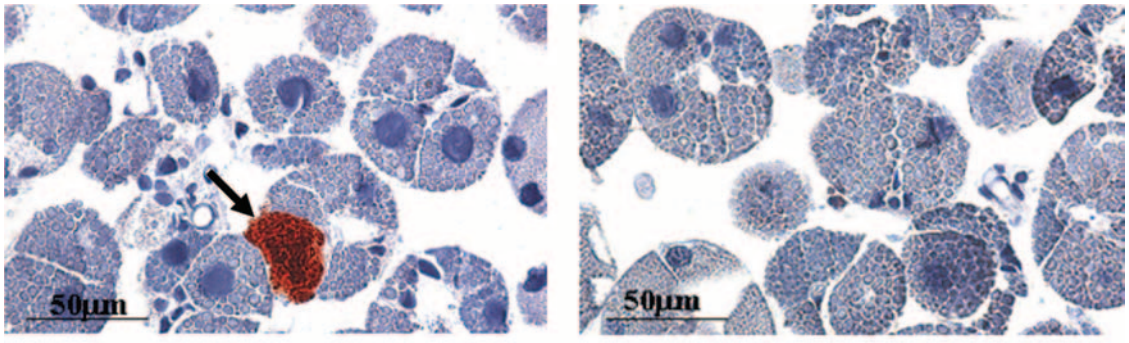

FIG. 2. Presence of *Babesia equi* within salivary gland acini of *Rhipicephalus microplus* males. (a) Infected *R. microplus* salivary glands probed with anti-*Babesia equi* MAb 36/253.21. An arrow indicates a *B. equi* colony within the acini of tick salivary gland tissue. (b) Same tissue shown in panel A probed with isotype-matched control MAb 18.185.

adult male *R. microplus* ticks can acquire *B. equi* parasites by feeding on an infected horse and then, following interhost movement, transmit the parasite to a naïve horse.

Similar to that of other vector-borne apicomplexan parasites, transmission of *B. equi* parasites requires the initial invasion of the midgut epithelium followed by the development of specific parasite stages within the tick, culminating in the replication and development of infective sporozoites in the salivary gland (10, 15, 16). The efficiency of the initial invasion by and establishment of *B. equi* parasites within adult male *R. microplus* ticks was affected by the level of parasitemia during acquisition feeding. However, once established in the tick, *B. equi* parasites replicated to similar levels (10^4 to 10^5 parasites/pair of salivary glands) at the time of transmission, independent of the parasitemia level during initial acquisition feeding. Although we did not design the experiment to compare the efficiencies of transmission between ticks that had been initially acquisition fed on acutely or chronically infected reservoir hosts, the ability of two infected adult male *R. microplus* ticks that had been acquisition fed on horses with chronic parasitemia to transmit *B. equi* parasites to a naïve horse (Table 1) is consistent with the finding that persistently infected horses are important reservoirs for transmission (20).

Within the tick salivary gland, *B. equi* parasites preferentially colonize the granular acinar cells (Fig. 2), and the transmission by infected ticks with $\geq 10^4$ *B. equi* parasites/pair of salivary glands (Table 1) indicates that this level of parasite replication within the acini exceeds the threshold for transmission. However, the mechanism by which *B. equi* parasites, as well as other parasites in the genus *Babesia*, develop infectivity at the time of transmission is poorly understood. The evidence presented here that two paralogs, EMA-1 and EMA-2, were expressed at levels in salivary glands significantly different from those expressed by intraerythrocytic parasites is the first evidence of differential surface protein expression in *B. equi*. Whether this differential expression has any functional role in infectivity or is simply a stage-specific marker is unknown at present.

In contrast to the intrastadial transmission mode demonstrated with adult male *R. microplus* ticks and the previously documented transstadial transmission (20), the hypothesis that vertical passage of *B. equi* parasites would allow transmission by the subsequent *R. microplus* generation was rejected. The experimental design followed that used to quantify successful transovarial transmission of *Babesia bovis* with the same colony of *R. microplus* ticks (7, 8). In *B. bovis* studies, the percentage of adult female *R. microplus* ticks that acquired infection (as detected by analysis of hemolymph) was positively correlated with the parasitemia level during acquisition feeding, with the highest infection rate in ticks allowed to feed on splenectomized calves (7). Correspondingly, we allowed adult female *R. microplus* ticks to feed on splenectomized horses with a peak parasitemia of $>10^9$ *B. equi* parasites/ml. Identification of hemolymph-positive females allowed the collection of eggs from those most likely to have passed *B. equi* parasites transovarially. Although vertical passage of *B. equi* parasites to the egg was detected, in agreement with a previous report (2), there was no transmission by any of the three tick stages (larval, nymphal, or adult) of the subsequent generation. This does not reflect simply a lack of initial acquisition of *B. equi* parasites by the adult female tick or a failure of transovarial passage, since $\geq$10,000 tick larvae derived from PCR-positive eggs failed to transmit. This result is in marked contrast to *B. bovis* transmission, where 12 to 48% of the offspring tick larvae are infected and uniformly transmit *B. bovis* to naïve calves (7). Vertical transmission requires infection within the ovary, passage via the egg to the next generation, and subsequent development of infectivity within the salivary gland of the progeny (7, 8). Our data suggest that while the first two steps may be relatively inefficient, there is also a lack of infectivity of the larvae derived from infected eggs. The lack of transmission, even when adult female *R. microplus* ticks were acquisition fed on horses with high levels of parasitemia and positive egg masses were selected for rearing larvae, indicates that this intergenerational mode of transmission is, at best, very inefficient and unlikely to be of epidemiologic significance. Whether this holds true for the other known or putative vector tick species shown to pass *B. equi* parasites transovarially is unknown and awaits definitive transmission data (1, 9).

In summary, these studies establish that, in addition to the previously documented transstadial transmission (20), intrastadial transmission by adult male *R. microplus* ticks is a mechanism of transmission for *B. equi* parasites and should be targeted for control. In addition, the ability of *R. microplus* to acquire *B. equi* parasites from horses with low-level parasitemia and to subsequently transmit the parasites with a minimal number of infected ticks indicates the importance of accurately detecting persistently infected horses despite the absence of clinical disease.

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